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Primer Note

Isolation and characterization of microsatellite loci from Amur honeysuckle, *Lonicera maackii* (Caprifoliaceae)[1]

Oscar J. Rocha[2,3], Erin McNutt[2], and Kelly Barriball[2]

[2]Department of Biological Sciences, Kent State University, Kent, Ohio 44424 USA

- *Premise of the study: Lonicera maackii* (Caprifoliaceae) is one of the most problematic invasive shrubs in forests of the eastern United States. Microsatellite markers could serve to test putative source-sink relationships among populations to determine whether invasions progress along expanding fronts or through long-distance dispersal events followed by local expansion.
- *Methods and Results:* Eleven microsatellite loci were developed for Amur honeysuckle using a modified magnetic bead protocol. Six loci were screened across 158 individuals from seven populations and were shown to be variable, with the number of alleles per locus ranging from seven to 16. Observed heterozygosity ranged from 0.655 to 0.757. Five additional loci were screened using 68 individuals from three different populations, with seven to 12 alleles per locus and observed heterozygosity ranging from 0.682 and 0.831.
- *Conclusions:* These microsatellite markers will help to elucidate the genetic structure and patterns of dispersal of *L. maackii* in its invasive range.

Key words: Caprifoliaceae; gene flow; *Lonicera maackii*; microsatellites; PCR; population structure.

Amur honeysuckle, *Lonicera maackii* (Rupr.) Maxim. (Caprifoliaceae), is a large, upright shrub native to the Russian Far East, China, Korea, and Japan; it was first introduced into the United States in 1898 and is currently one of the most problematic invasive shrubs in forests of the eastern United States (Luken and Thieret, 1996). Additional introductions from Asian populations and accessions from European botanical gardens were made by the U.S. Department of Agriculture at least 14 times over the following 86 yr (Luken and Thieret, 1996). The Soil Conservation Service developed and promoted cultivars for wildlife habitat and food, screens, and wind barriers (Sharp and Belcher, 1981; Luken and Thieret, 1996), and these cultivars have been widely used in horticulture. Naturalized populations were first described in the late 1950s and now occur in Ontario and nearly every U.S. state east of the Mississippi (Hutchinson and Vankat, 1997).

Several studies indicate that native forest stands where *L. maackii* has invaded have lower species richness and lower seedling and sapling density in both herbs and trees, lower tree basal area growth (Hutchinson and Vankat, 1997), reduced survival and seed production of forest annuals (Gould and Gorchov, 2000), reduced growth and reproduction of perennials (Miller and Gorchov, 2004), and lower survival of tree seedlings (Gorchov and Trisel, 2003). Despite the negative impacts of *L. maackii* on native plants in invaded forests, little is known about its dispersal patterns and range expansion. The study of its genetic structure could provide insight to determine whether invasions progress through long-distance dispersal events followed by local expansion. Here, we describe 11 polymorphic microsatellite loci developed to study the genetic structure of *L. maackii*.

METHODS AND RESULTS

***Leaf tissue collection*—**Leaf samples of *L. maackii* were collected from four to 31 plants from 10 different woodlots in Darke, Preble, and Butler counties, Ohio. Leaf tissue was collected on dry ice prior to storage at −40°C until DNA extraction. Total genomic DNA was extracted from leaves of each honeysuckle plant using the cetyltrimethylammonium bromide (CTAB) protocol described by Doyle and Doyle (1987).

***Development of microsatellite markers*—**The microsatellite markers were developed using a modified magnetic bead protocol (Glenn and Schable, 2005). Genomic DNA was digested from a concentrated sample of *L. maackii* plants using *Hae*III/*Psh*A1 restriction enzyme (Invitrogen, Carlsbad, California, USA). Two linkers were ligated onto the digested genomic DNA (M28 5′-CTCTTGCTTGAATTCGGACTA-3′ and M29 5′-pTAGTCCGAATTCAAGCAAGAGCACA-3′), and M28 was used as a primer for subsequent PCR. The digested genomic DNA was amplified in multiple PCR reactions and concentrated to gain enough DNA for the following bead hybridization process. Six arbitrary repeat motifs with a biotin label on the 5′ end were chosen as probes for the bead hybridization reactions (TC_{12}, AC_{12}, AAG_7, AAC_7, $GATA_6$, $CATA_6$). After the hybridization, the selected fragments were isolated from the rest of the genomic DNA using streptavidin-coated magnetic beads to enrich for genomic DNA containing the selected repeats. The repeat-enriched DNA was then ligated into a pGEM-T vector (Promega Corporation, Madison, Wisconsin, USA) and cloned into electrocompetent *E. coli* cells. Positive clones were sequenced

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[3]Author for correspondence: orocha@kent.edu

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TABLE 1. Oligonucleotide primer sequences, repeat motifs, number of alleles, product sizes, and annealing temperatures for each of the 11 microsatellite loci isolated for *Lonicera maackii*.

Locus	GenBank accession no.	Repeat motif	Primer sequences (5′–3′)	No. of alleles	Allele size range (bp)	T_a (°C)
Maack-Di1 F	KF463290	$(AG)_{36}$	D3-CTACCTGTCCCTTTCTTTG	9	188–205	55
Maack-Di1 R			GAGGGTAAAAAGAGAAGAAGG			
Maack-Di3 F	KF463291	$(CA)_9$	D3-AAAAGGCAAAGAAGCTCTTGGCA	7	216–228	55
Maack-Di3 R			AGAAAAGAAGTCAGACTCTGCA			
Maack-Di4 F	KF463292	$(CT)_{19}$	D4-CTCATTCAGTCAAGTCCAAGT	11	125–147	55
Maack-Di4 R			CGATGCTACATCATAATTAACAG			
Maack-Di5 F	KF463293	$(GA)_{23}$	D3-CAAATTCCCTGTTATTCAAAGG	10	177–195	55
Maack-Di5 R			CCTGTATGTGCTGATAATCAT			
Maack-Di19 F	KF463294	$(CT)_{12}$	D4-CGTGTTCCCCTTCTCTCACT	16	236–272	60
Maack-Di19 R			CGGGGCTGCTTATCTTCTTC			
Maack-Tri1 F	KF463296	$(CTT)_{20}$	D3-CCGTTTCATAAGTTAATAGAGGT	7	152–164	55
Maack-Tri1 R			TTGAGAGCCCTTCTGCGTTC			
Maack-Tri11 F	KF463300	$(GTT)_7(CTT)_{13}$	D4-GCAAGTATGGCTGGGTTGAT	11	129–162	55
Maack-Tri11 R			GCTCTTCTTGTTGTCCGTGA			
Maack-Tri8 F	KF463299	$(GAA)_{15}$	D3-TCAAACGAGCTCCTAGATTGTAAA	13	141–177	55
Maack-Tri8 R			GTTAGCGTGTTGCGTTCACT			
Maack-Tet20 F	KF463297	$(GTAT)_{17}$	D4-CACGTATGGAACACGAAAAGA	6	152–167	55
Maack-Tet20 R			TGTGAAGATATTTGTCATCACGTTT			
Maack-Tet21 F	KF463298	$(GTAT)_6(GTAT)_7$	D4-GCCTCCACCGATCTACTTCA	11	152–220	55
Maack-Tet21 R			TCGGACGGTCGTTATGTGTA			
Maack-Tet22 F	KF463295	$(GTAT)_{16}$	D4-CATGAAGCCATTCGAAATCA	15	152–220	55
Maack-Tet22 R			CATGAAGCCATTCGAAATCA			

Note: T_a = annealing temperature.

on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, California, USA). Sequences were obtained using universal M13 forward (F) and reverse (R) primers employing ABI PRISM BigDye Terminator sequencing kits (Applied Biosystems). Sequences containing repeat motifs and sufficient flanking sequences were used to design primers with Primer3 software (Rozen and Skaletsky, 2000). All sequences were deposited in GenBank (Table 1). Twenty primer pairs were screened on 3% agarose gels against 12 samples from different locations to identify polymorphic markers. Of the 20 markers that were initially screened, 11 were determined to be polymorphic and we then obtained these markers with fluorescent dye labels (Table 1). All forward primers were labeled with WellRED fluorescent dyes (D4 or D3; Integrated DNA Technologies, Coralville, Iowa, USA). Once PCR reactions were optimized, samples from individual plants taken from 10 populations were used to determine the diversity of these loci.

Voucher specimens of the samples used for the development of these microsatellite loci were deposited in the herbaria at Miami University (MU 284343) and Kent State University (KE 66371). DNA samples are maintained in the first author's laboratory at Kent State University, and can be made available upon request.

***PCR amplification and fragment analysis*—**PCR reactions were performed using a PTC-200 thermocycler (MJ Research, Watertown, Massachusetts, USA) in 20-μL solution containing 5 μL of genomic DNA, 10 mM Tris buffer (pH 8.0), 10 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primer of each primer, and 1 unit of *Taq* polymerase (Fermentas International Inc., Burlington, Ontario, Canada). The PCR program used included an initial step of 1 min of denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final extension cycle of 5 min at 72°C. Genotyping was conducted using capillary electrophoresis on an automated genetic DNA analysis system (CEQ 8800; Beckman Coulter, Fullerton, California, USA). Four microliters of PCR product were mixed with 28 μL of formamide and 0.4 μL of 400-bp DNA size standard (GenomeLab, Beckman Coulter) for capillary electrophoresis. Fragments were identified on the basis of their size and according to their mobility in relation to the size standard using a cubic function.

Electrophoresis data were collected automatically using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter). Once all of the data scoring was complete, random samples were reamplified and rerun to assess reproducibility and confirm the scoring and allele sizes.

***Genetic data analysis*—**Genetic diversity was quantified for all loci by the mean number of alleles per locus (A), the effective number of alleles per locus (A_e), observed heterozygosity (H_o), and Nei's expected heterozygosity (H_e) for each locus and averaged across all loci using the program POPGENE 1.31 (Yeh et al., 1999). All loci examined were polymorphic across all populations, and showed high levels of allelic diversity. A total of 73 and 32 different alleles were found for the first six and second five loci, respectively. The average A in each *L. maackii* population averaged 7.07 and 6.27 (Tables 2, 3). Average A_e was lower than A across all populations (A_e = 4.41

TABLE 2. Geographical coordinates, mean number of alleles (A), mean effective number of alleles per locus (A_e), mean observed heterozygosity (H_o), Nei's mean expected heterozygosity (H_e), and loci deviating from Hardy–Weinberg equilibrium (HWE) for 10 populations of *Lonicera maackii* from southwestern Ohio, USA, from 158 individuals taken from seven populations in the first six loci (Maack-Di3, Maack-Di4, Maack-Di19, Maack-Tetra21, Maack-Tetra22, and Maack-Tri11).[a]

Population	Sample size	Geographical coordinates		A	A_e	H_o	H_e	Loci deviating from HWE*
CX	18	39°47′11.19″N	84°44′27.70″W	7.33 (1.12)	4.58 (0.720)	0.796 (0.095)	0.753 (0.038)	Di4
OHR	29	39°30′31.00″N	84°42′55.07″W	8.83 (2.33)	5.48 (1.250)	0.659 (0.109)	0.804 (0.029)	Di3, Di4
SOLL	27	39°57′17.18″N	84°43′11.50″W	7.33 (0.76)	4.62 (0.390)	0.637 (0.068)	0.776 (0.019)	Di4, Tet21, Tet22
HA	31	39°50′11.19″N	84°50′54.37″W	7.00 (1.53)	4.35 (0.950)	0.751 (0.660)	0.710 (0.059)	Tet21
JM	27	40°01′55.80″N	84°44′26.15″W	7.50 (1.17)	3.85 (0.530)	0.715 (0.096)	0.712 (0.042)	Di4, Di19, Tet21, Tet22
NMILLS	8	39°58′24.91″N	84°43′51.86″W	5.50 (0.62)	4.06 (0.570)	0.845 (0.062)	0.728 (0.039)	
NP	18	39°50′28.67″N	84°50′58.73″W	6.00 (0.57)	3.90 (0.740)	0.648 (0.083)	0.693 (0.055)	Di3, Tri8, Tet21
Overall	158			7.07 (0.40)	4.41 (0.24)	0.722 (0.032)	0.739 (0.016)	

[a] Standard errors are shown in parentheses.

* Bonferroni correction for multiple tests ($\alpha = 0.05$) was made according to Weir (1996).

TABLE 3. Geographical coordinates, mean number of alleles (A), mean effective number of alleles per locus (A_e), mean observed heterozygosity (H_o), Nei's mean expected heterozygosity (H_e), and loci deviating from Hardy–Weinberg equilibrium (HWE) for 10 populations of *Lonicera maackii* from southwestern Ohio, USA, from 68 individuals taken from three populations in the remaining five loci (Maack-Tri1, Maack-Tri11, Maack-Tetra20, Maack-Di1, and Maack-Di5).[a]

Population	Sample size	Geographical coordinates		A	A_e	H_o	H_e	Loci deviating from HWE*
MILL	14	39°55′20.20″N	84°40′45.79″W	5.80 (0.73)	3.79 (0.59)	0.831 (0.053)	0.706 (0.049)	Di5, Tri1
Woodlot 379	32	39°47′42.00″N	84°46′53.40″W	6.40 (0.93)	3.58 (0.43)	0.781 (0.062)	0.706 (0.032)	
WHIN	22	39°48′38.50″N	84°41′49.50″W	6.60 (0.93)	3.98 (0.65)	0.682 (0.085)	0.731 (0.058)	Di5, Tet20
Overall	68			6.27 (0.47)	3.78 (0.30)	0.765 (0.040)	0.708 (0.025)	

[a] Standard errors are shown in parentheses.

* Bonferroni correction for multiple tests ($\alpha = 0.05$) was made according to Weir (1996).

and $A_e = 3.78$). H_o and H_e were also high across all populations. *F*-statistics indicate that these populations have low levels of inbreeding (mean fixation index [F_{IS}] = 0.0183). Analysis of molecular variance (AMOVA) revealed that 92% of the variation arises from within-population differences. Geographic coordinates for all woodlots where the samples were collected are given in Tables 2 and 3.

Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were determined using POPGENE 1.31. Our analyses revealed significant deviations from HWE in eight of the 10 populations for at least one locus (Tables 2, 3). Given that these populations were recently established and most likely received seeds from multiple nearby invaded woodlots, it is reasonable to expect that they will not be in equilibrium. Moreover, we also found linkage disequilibrium for multiple paired comparisons in many populations, but it is difficult to make inferences about linkage disequilibrium given the deviations from HWE observed in these populations. Furthermore, we found that pairs of loci showing linkage disequilibrium were not consistent across populations. These loci should now be tested with samples from parent–offspring pairs to ensure that these markers behave as expected.

CONCLUSIONS

The results described here allow preliminary inferences about the genetic structure of the invasive shrub *L. maackii* using newly developed microsatellite markers. Our results show high levels of genetic diversity among populations in southwestern Ohio. The high levels of heterozygosity and low F_{IS} values observed in these populations indicate that there is little inbreeding, suggesting that this species is predominantly an outcrosser. Moreover, a significant proportion of the genetic diversity (8%) was found among populations, a level of genetic differentiation consistent with the range observed for short-lived perennials (Hamrick and Godt, 1990). These findings indicate that these microsatellite markers are a useful tool to elucidate the genetic structure and dispersal patterns of *L. maackii* in its invasive range.

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